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(54) **Process for purifying hepatitis a virus (HAV), virus thus purified and vaccine compositions containing it.**

(57) A process is described for the purification of the hepatitis A virus, which allows to obtain with good yields a pure product, in which organic material collected by centrifugation after lysis of the culture cells is submitted to gel filtration and successively to ion exchange chromatography.

EP 0 522 291 A1

Invention Field

A process is described for the purification of the hepatitis A virus (HAV) in which the material from the culture cells, after cell lysis and centrifugation, is submitted to gel filtration and the thus obtained eluate is submitted to ion exchange chromatography.

State of the Art

The hepatitis A virus (HAV) is a icosahedral morphology Picornavirus with 32 capsomeres on its surface, which presents four important structural VP1 polypeptides with a molecular weight MSW 30.000-33.000, VP2 24.000-27.000, VP3 21.000-23.000, VP4 7.000-14.000.

Said four proteins are the ones responsible for the antigenic virus power and are therefore the ones which the purification processes tend to put in evidence and to isolate in order to obtain, with a good degree of purity, an inactivated vaccine.

The purification processes seek to eliminate cellular contaminants and growth factors which are employed in the virus production process.

Various methods for the partial virus purification, both for vaccination and for virus characterization purposes, have been described. For example, through the virus sedimentation by means of a 20% sucrose pad and successive centrifugation in a cesium chloride gradient [P.J. Provost et al. J. of Medical Virology 19, p.23-31 (1986)], through ammonium sulphate precipitation and sedimentation with a 20% sucrose pad and cesium chloride gradient [Flehmig B. et al., J. of Medical Virology 22, p. 7-16 (1987)], with a lysis buffer, freezing, defrosting, sonication to set the virus free, ultrafiltration with tangential flow and purification in cesium chloride gradient [Flehmig et al., The Lancet, May 13, p.1039 (1989)], through various clarification cycles and successive freeze or chloroform extraction followed by gel filtration, ion exchange chromatography and purification in cesium chloride gradient [S.A. Locarnini, Intervirology 10, 300-308 (1978)].

All the above mentioned processes employ, at least in one step, ultracentrifugation systems and high cost materials such as cesium chloride, and therefore, although yielding excellent results on a small scale, are hardly suitable for an industrial production, in which process duration, costs and availability of suitable personnel have to be taken into consideration.

In the European Patent Application EP-A-302692 a process for the purification of hepatitis A virus is described, which employs sonication for the cell lysis, followed by extraction with organic solvents and successive concentration, chromatog-

raphy on anion exchange resins and, finally, gel filtration chromatography. Also this process, particularly in view of the use of sonication and of organic phase extractions and concentrations, presents, on an industrial scale, certain operative difficulties.

Detailed description of the invention

The process according to the present invention allows to obviate the mentioned drawbacks and is therefore a valid contribution to the purification on an industrial scale and the production of a purified HAV virus suitable for the use as a vaccine.

Diploid human cells MRC-5 designated by the World Health Organization as suitable for the production of vaccines for human use, cultivated and collected according to conventional techniques, were used.

The cells were infected with HAV at the 30th passage. After 21 days incubation, the cell substrate was washed with PBS-A to eliminate as much as possible the fetal bovine serum present in the culture medium and indispensable for the substrate.

The infected cells were taken up with trypsin EDTA following traditional methods and re-suspended in isotonic buffer (Tris 10 mM, NaCl 10 mM, pH 7.5), this causing cell lysis and therefore setting the virus free, and frozen.

At the time of purification the material is defrosted and treated with 2% Triton-X-100 for 30 min. at room temperature, stirring about every 5 min.; the material is then collected by centrifugation, so as to remove the cell fragments; this treatment allows the solubilization of membrane lipids with which the virus is strictly associated. The next step is gel filtration, employing gel filtration beds of both agarose and dextrane, e.g. Sepharose C1-4B (Pharmacia) equilibrated in TNE buffer (Tris 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7.2-7.6), containing 0.1-0.2% Triton-X-100 or glycine buffer 0.1 M with 0.2% deoxycholate, pH 8.5. The eluted material is collected in 20 ml fractions which are tested for the presence of HAV by an ELISA assay. With this passage, yields of 85-95% are obtained with an approximate eight-fold purity increase (30-50 µg virus per mg of protein).

The eluate obtained in the preceding step is then submitted to ion exchange chromatography employing anion exchange resins, such as e.g. DEAE Sepharose CL-6B equilibrated in TNE containing 0.1%-0.2% Triton-X-100; in these conditions the virus is adsorbed on the bed while part of the contaminants are not retained.

After washing the column with TNE, to eliminate the detergent, elution is performed decreasing the pH and increasing the ion strength. To this

end a phosphate buffer may be employed with a continuous pH gradient from 7.4 to 4 and ionic strength from 0 to 0.3 M NaCl. The yield in this second step is of the order of 50% with respect to the preceding step and the purity of the collected virus is increased 6 to 10 times (with an average virus contents of 70% on the total protein). The thus purified material is filtered on a membrane of 0.22 μ m porosity and inactivated with 1:2000 formalin at 35°C for 5 days under continuous stirring.

During the inactivation period, disaggregating treatments are performed, the 2nd day the material is sonicated at 50-60 W 1 sec/ml. The third day the material is filtered on a 0.22 μ m membrane and L-lysine.HCl 25 mM is added. After inactivation, the suspension is dialyzed against PBS A (1:100 v/v) for about 36 hrs, with an intermediate buffer substitution. After dialysis, the material is submitted to a sterilizing filtration and the product undergoes all the required controls: sterility, pyrogenicity, inactivation, antigenicity, pH, stability, residual formalin.

Experimental Section

MRC-5 cells at the 30th passage in rotating 850 cm³ bottles are infected with HAV (strain LSH/S ATCC VR 2266) at a 0.5 MOI.

After a 20 day incubation period, the cellular substrate is washed 3 times with serum-free medium maintaining the last washing overnight. The following day the cells are removed with tripsine-EDTA following traditional methods, and suspended again in isotonic buffer (Tris 10 mM, NaCl mM, pH 7.5) 1 ml for each 100 cm² cell culture and frozen.

60 ml of the frozen suspension, deriving from approximately 5.700 cm² culture are defrosted and treated with a non ionic detergent (2% Triton-X-100) for 20 to 30 minutes at room temperature under moderate stirring every 5-10 minutes.

The sample is centrifuged at 400 g for 10 minutes while cooling to remove cellular fragments. The supernatant is purified through gel filtration on a agarose resin (Sephacrose CL4B Pharmacia)-column 5 x 90 cm (K 50/100 column, Pharmacia) equilibrated with Tris 10 mM, NaCl 150 mM, EDTA 1 mM buffer, pH 7.4, containing 0.1% Triton-X-100 at a 75 ml/h flow rate. The eluted material is collected in 20 ml fractions which are tested for the presence of HAV by a ELISA assay. The HAV containing fractions are collected, obtaining approximately 400 ml. This material is further purified by ion exchange chromatography seeding about 200 ml, at a flow rate of 100 ml/h on a anionic resin (Sephacrose CL6B Pharmacia) column 5 x 5 (column XK 50/30 Pharmacia) which had previously been equilibrated in Tris 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7.4 buffer containing 0.1% Triton-X-100. Under such conditions the virus is adsorbed

on the matrix. The matrix is washed with Triton-X-100 free buffer to remove the detergent and the virus is eluted at a flow of approximately 160 ml/h, applying a continuous pH gradient and ionic strength, starting from pH 7.4 and NaCl 0 mM to pH 4 and NaCl 0.3 M. The eluted material is collected in fractions of about 10 ml and the fractions found positive for the presence of HAV at a ELISA assay are put together.

A virus of 70% purity is thus obtained. The thus purified material is filtered on 0.22 μ m porous membrane and inactivated with formalin 1:2000 at 35°C for 5 days under continuous stirring. During the inactivation period, disaggregation treatments are performed: on the 2nd day the material is sonicated at 50-60 W per 1 second/1 ml; on the 3rd day it is filtered on a 0.22 μ m membrane and L-lysine.HCl 25 mM is added.

After inactivation, the suspension is dialyzed against PBS-A (KCl 2.7 mM, KH₂PO₄ 1.5 mM, NaCl 137 mM, NaH₂PO₄ 8.1 mM, pH 7.4) in a 1:100 v/v ratio for 36 hours with an intermediate buffer substitution. After dialysis, the material undergoes a sterilizing filtration and is then submitted to the usual controls for sterility, pyrogenicity, inactivation, antigenicity, pH, stability and residual formalin.

Claims

1. Process for purifying the hepatitis A virus (HAV) in which the material from the culture is submitted, after cell lysis and centrifugation, to gel filtration, and the thus obtained eluate is submitted to ion exchange column chromatography.
2. Process according to claim 1 in which the gel filtration is performed with agarose or dextrane matrices.
3. Process according to claim 2 in which the gel filtration matrix employed is Sepharose CL-4B.
4. Process according to claim 1 in which the gel filtration matrix is equilibrated with a pH 7.2-7.6 detergent containing buffer.
5. Process according to claim 4 in which the detergent containing buffer consists of Tris 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7.4.
6. Process according to claim 5 in which said buffer contains an amount of 0.1-0.2% of Triton-X-100 as detergent.
7. Process according to claim 1 in which the ion exchange chromatography is performed on

DEAE Sepharose CL-6B.

8. Process according to claim 7 in which DEAE Sepharose CL-6B is equilibrated with a pH 7.2-7.6 detergent containing buffer.

9. Process according to claim 8 in which the detergent containing buffer consists of Tris 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7.4.

10. Process according to claim 9, in which said buffer contains an amount of 0.1-0.2% of Triton-X-100 as detergent.

11. Process according to claim 1, in which the ion exchange chromatography column is eluted decreasing the pH and increasing the ionic strength.

12. Process according to claim 11, in which a phosphate buffer with continuous pH gradient from 7.4 to 4 and ionic strength from 0 to 0.3 M NaCl is employed as eluent.

13. Process according to claim 11 in which the virus contained in the eluate is successively inactivated with 1:2000 formalin.

14. Process for purifying the hepatitis A virus (HAV) comprising the following steps:

a) MRC-5 cells infected by strain LSH/S (ATCC VR 2266) HAV virus are cultivated and collected;

b) the cells are submitted to lysis with 2% Triton-X-100 and centrifuged;

c) the collected material is submitted to gel filtration on Sepharose CL-4B equilibrated with pH 7.4 Tris 10 mM, NaCl 150 mM, EDTA 1 mM, containing 0.1% of Triton-X-100;

d) the eluate from (c) is submitted to anion exchange chromatography on DEAE Sepharose CL-6B equilibrated with pH 7.4 Tris 10 mM, NaCl 150 mM, EDTA 1 mM, containing 0.1% of Triton-X-100;

e) the HAV virus adsorbed on the column in (d) is eluted in a continuous pH gradient employing phosphate buffer with a continuous gradient from 7.4 to 4 and a ionic strength gradient from 0 to 0.3 M NaCl

f) the purified virus obtained in (e) is inactivated in 1:2000 formalin.

15. Hepatitis A virus (HAV) strain LSH/S deposited as ATCC VR 2226.

16. HAV virus purified and inactivated, obtained according to the process claimed in claim 14.

17. The use of the inactivated virus according to claim 16 for the preparation of vaccine compositions against hepatitis A.

18. Vaccine compositions containing the virus according to claim 16.

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EUROPEAN SEARCH REPORT

Application Number

EP 92 10 9524

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,X	EP-A-0 302 692 (MERCK & CO. INC.) * the whole document * ---	1-3, 16-18	C12N7/02 C12N7/00 A61K39/29
A	EP-A-0 171 765 (JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE) * the whole document * ---	1,2,13, 14	
A	EP-A-0 327 801 (DEUTSCHES PRIMATENZENTRUM GESELLSCHAFT MBH) * abstract; claims 1-5; example 5 * * claim 4; example 4 * ---	5,9	
A	JOURNAL OF CHROMATOGRAPHY vol. 297, 1984, AMSTERDAM NL pages 101 - 109 Welling GW; Nijmeijer JR; van der Zee R; Groen G; Wilterdink JB; Welling-Wester S 'Isolation of detergent-extracted Sendai virus proteins by gel-filtration, ion-exchange and reversed-phase high-performance liquid chromatography and the effect on immunological activity.' * the whole document * ---	4,5,8,9, 10,14	
A	EP-A-0 171 086 (JURIDICAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE) * examples 1-5 * -----	12	
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 20 OCTOBER 1992	Examiner GURDJIAN D.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons * : member of the same patent family, corresponding document			